International Journal of Applied and Natural Sciences (IJANS)
ISSN 2319-4014
Vol. 2, Issue 4, Sep 2013, 15-22

International Academy of Science, Engineering and Technology
Connecting Researchers; Nurturing Innovations

CHARACTERIZATION OF BACTERIA ISOLATED FROM *PENAEUS MONODON* DIGESTIVE TRACT WITH CYTOTOXIC EVALUATION

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ABSTRACT

A gram negative, motile, lactose fermenting, *Pseudomonas aeruginosa* was isolated from the dissected digestive tract of *Penaeus monodon* by plating the sample onto an agar solidified LB medium. The optimum growth of the bacteria was observed at pH 7.5 and at temperature 30^0C. Cytotoxic effect of bacteria and bacteriocin was tested on *Artemia salina* through LC_{50} to evaluate whether they have any beneficial effect or not. LC_{50} for bacteria was 205.9645 µl (O.D.=600) and the regression equation was Y = -1.064911 + 2.621199 X, while the 95% confidence limits are 143.3878 to 295.8508 µl for 12h exposure. Whereas for bacteriocin LC_{50} was 65.65591 µl and the regression equation was Y = 2.166407 + 1.559255 X, while the 95% confidence limits are 25.40323 to 69.691 µl for 6h exposure only. So, the bacteriocin has adverse effect on *Artemia salina* and the bacteria showed resistant against Gentamycin, Ceftazidime and Nalidixic acid and the MIC value was 12.5 µg/ml against Gentamycin.

KEYWORDS: Bacteriocin, Cytotoxicity, $LC_{50,}$ *Penaeus monodon*, *Pseudomonas aeruginosa*

INTRODUCTION

Artemia salina is one of the most valuable test organism available for ecotoxicity testing and research done so far allows us to state that it is possible to sustain several options related to *Artemia salina* use in toxicology and ecotoxicology. (Bruno S. Nunes *et al.,* 2006) and bacteriocins are bacterially produced peptide antibiotics with the ability to kill a range of bacteria (Cleveland *et al.,* 2001). Both gram-positive and gram-negative bacteria have produced them. Bacteriocin-mediated antagonism is believed to occur in virtually any niche colonized by bacteria. They are heterogeneous compounds with variable molecular weight, biochemical properties, inhibitory spectra and mechanism of actions (Sullivan *et al.*,2002). The production of bacteriocin or bacteriocin-like substances has been described for *B. coagulans, B. brevis, B. lichniformis, B. cereus, B. subtilis, B. amyloli-quefaciens* and other *Bacillus* species (Lisboa *et al.,* 2006). The purpose of this study was isolation and characterization of bacteria from *Penaeus monodon* digestive tract and to evaluate it's bacteriocin on *Artemia salina*, wheather it can effect on prawn at high concentration or not.

MATERIALS AND METHODS

A single bacterial colony was isolated aseptically by plating from the dissected digestive tract of the collected *Penaeus monodon* onto an agar solidified LB medium. The plates were incubated at 37°C for 24 hours and bacterial colonies were found to grow on the medium.

IDENTIFICATION OF THE BACTERIAL ISOLATE

Microscopic Observation

Microscopic examination of bacterial cells was done after gram staining and additional morphological, physiological tests were also conducted.

16S rRNA Gene Sequencing

Genomic DNA of bacterial isolate was isolated according to the previously described method (Sambrook *et al*, 1989). Gene fragments specific for the highly variable region of the bacterial 16S rRNA gene was amplified by PCR using universal PCR primer as described by Loffler *et al*.,(2000) (Sigma, USA) in a thermal cycler (MJ Research Inc., Watertown, USA). The sequence of the universal forward 20mer 5' GTTGTAAAACGACGGCCAGT 3'. Universal Reverse 20mer 5' CACAGGAAACAGCTATGACC 3'.

The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. Amplified 16S rRNA gene PCR products were purified using Strata Prep PCR purification kit (Stratagene, USA) according to the manufacturers protocol. Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABIPrism 310 genetic analyzer.

The chromatogram sequencing files were edited using Chromas 2.32. The homology of the 16S rRNA gene sequence was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to Gen Bank database using the BLASTN (http://www.ncbi.nih.gov/BLAST/) algorithm.

Effect of Temperature and pH on Bacterial Growth

To determine the effect of pH on bacterial growth, culture medium was adjusted to pH 5.5, 6.5, 7.5 and 8.0. Incubation temperature was varied from 25^0C, 30^0C and 37^0C. Bacterial cell density of liquid culture was determined by measuring optical density at 620 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO).

Antibiotic Sensitivity Test

Different types of antibiotic discs were used to check the resistance or sensitivity of the bacterial isolate. The discs were placed on agar plate with bacterial culture. The plates were then incubated at their respective temperature for overnight. After 16 hours of incubation, the diameter of clear zone around the discs were measured with the help of millimeter scale.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of gentamycin was determined by turbidimetric method against isolated bacteria. In this method, a large number of autoclaved test tubes were used and each test tube contained 10 ml of sterilize MS broth media and test organisms. Various concentrations of gentamycin were applied to the MS broth media and the microorganisms were incubated at 28 ^{0}C for 72 hours.

Plasmid Extraction

The plasmid DNA of the microorganism was isolated according to Birnboim and Doly method (1979) without any multification.

Plasmid Curing

Methods were taken from the review on plasmid curing by Trevors (1986). Ethidium bromide, acriflavin, SDS were used as curing agents. In addition, repeated subcultures in nutrient broth in the absence of appropriate carbon source were used as a curing strategy.

Cytotoxic Evaluation through *Artemia salina* (Brine Shrimp) Nauplii

Brine shrimp lethality bioassay (Jaki *et al*., 1999; Mayer *et al.*, 1982; McLaughlin and Anderson, 1988) is a recent development in the assay procedure of bacteriocin which indicates cytotoxicity as well as a wide range of pharmacological activities of the bacteriocin.

Several doses were selected by a pilot experiment and a final experiment was set up with 3 replications along with control for the detection of cytotoxic activity. The result was then subjected to probit analysis through probit mortility software.

RESULTS

Identification of Bacterial Isolate

The results of the microscopic observations and biochemical tests are presented in table 1 and 2. The 16SrRNA gene sequence were submitted to NCBI website and the BLAST query confirmed the isolate as *Pseudomonas aeruginosa*.

Table 1: Culture Media Dependent Characteristics and Microscopic Observation of *Pseudomonas aeruginosa*

Agar Plate	Characters	Results
Nutrient agar plate	Size	(1-2) mm
	Shape	rod
	Colour	Greenish
	Consistency	Sticky
	Opacity	Translucent
	Elevation	Raised
	Margin	Entire
Nutrient agar slant	Abundance of growth	Moderate
	Colour	Greenish
Nutrient broth culture		Uniform with fine turbidity
Microscopic observation	Gram staining	Gram-negative
	Motility	Motile

Table 2: Biochemical Tests of *Pseudomonas aeruginosa*

Sugar Utilization	
Carbone Source	***Pseudomonas aeruginosa***
Monossacharides	
Glucose	+
Arabinose	-
Dissacharides	
Sucrose	-
Lactose	-
Maltose	+
Fructose	+
Cellulose	-
Character	
Methyl Red (MR)	+
MacConkey agar	-
Mobility	+
Indole	-
Catalase test	+
Simmons citrate agar test	-

Effect of pH and Temperature on Bacterial Growth

The optimum growth of the *Pseudomonas aeruginosa* was observed at pH 7.5 and pH 5.5 restricted the bacterial growth and the optimum temperature for growth was 37^0C.

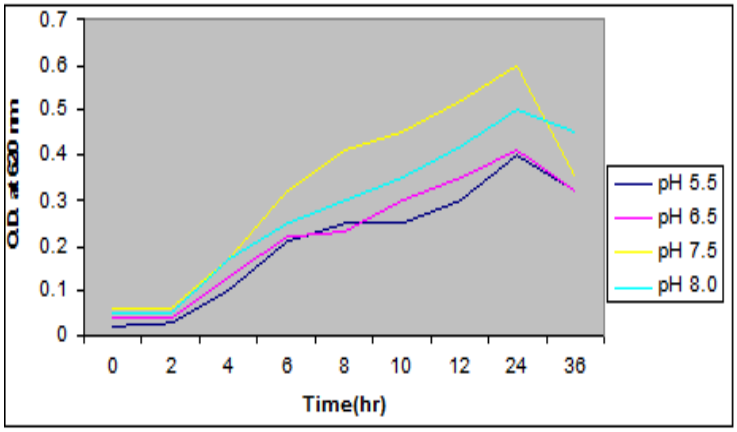

Figure 1: Effect of pH on Bacterial Growth

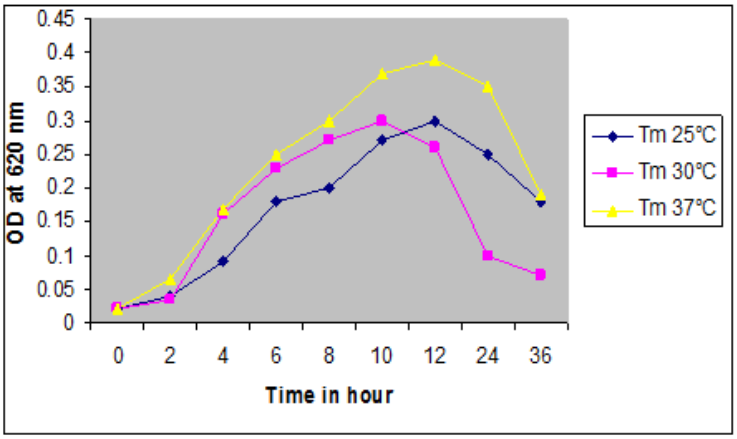

Figure 2: Effect of Temperature on Bacterial Growth

Antibiotic Resistance and MIC

The results of the antibiotic sensitivity test are presented in table 3. From the table, it was evident that the *Pseudomonas aeruginosa* was resistant to Gentamycin, Ceftazidime and Nalidixic acid.

Table 3: Antibiotic Sensitivity Tests

Antibiotics	Range of Antibiotics	R	S and I
Amoxicillin	13mm	-	I
Penicillin	22mm	-	S
Ceftazidime	9mm	R	-
Vancomycin	20mm	-	S
Gentamycin	10mm	R	-
Ciprofloxacin	30mm	-	S
Nalidixic acid	-	R	-

(5-10mm) = Resistance to antibiotic(R), (15-20mm) = Sensitive to antibiotic(S), (10-15mm) = intermediate resistance (I)

The MIC of Gentamycin against *Pseudomonas aeruginosa* was 12.5 µg/ml, demonstrating that low concentration of this antibiotic was required to inhibit the growth of this bacteria.

Plasmid Content of the Wild Type and Cured Bacterial Strain

To determine the possible role of plasmid DNA in case of toxicity, the plasmid content of wild type and cured strain was examined. The wild type bacterial strains were contained three DNA bands of plasmid. The cured strains of the bacteria were found to have lost the entire band of plasmid DNA. The loss of bands in the cured strain proved that the bands found on the gel belonging to the DNA carrying the gene(s) responsible for the toxicity, especially lethality. Thus,

the loss of plasmid bands with the treatment of ethidium bromide (100 µg/ml) suggested that there was strong correlation between the inability to attack *Artemia salina* with the lost plasmid bands.

This result strongly suggested that the gene(s) responsible for the ability to show toxic effect on aquatic organisms might be plasmid DNA mediated.

Cytotoxic Evaluation through *Artemia salina* (LC_{50})

Integration of different areas of scientific knowledge concerning biology, life cycle and environmental needs of *Artemia* is of crucial importance when considering the interpretation of results drawn from tests involving this genus (Na1ecz-Jawecki *et al.,* 2003). LC_{50} for bacteria was 205.9645 µl (O.D.=600) and the regression equation was Y = -1.064911 + 2.621199 X, while the 95% confidence limits are 143.3878 to 295.8508 µl for 12h exposure. Whereas for bacteriocin LC_{50} was 65.65591 µl and the regression equation was Y = 2.166407 + 1.559255 X, while the 95% confidence limits are 25.40323 to 69.691 µl for 6h exposure only. So, the bacteriocin had adverse effect on *Artemia salina* (Table 4). It can easily be predicted that the *Pseudomonas aeruginosa* can also severely affect *Penaeus monodon* due to aquatic organisms.

Table 4: LC_{50}, 95% Confidence Limits, Regression Equation and χ^2 Value of Dose Cytotoxicity Experiments of the Bacteriocin and Isolated Bacterium against *A. salina* with 06 and 12 Hours Exposure

Tested Sample	Exposure (hours)	LC_{50} Value (µl) (O.D.=620nm)	95% Confidence Limits		Regression Equation	χ^2 Value (df)
			Upper	Lower		
bacteriocin	06	65.65591	69.691	25.40323	Y = 2.166407 + 1.559255 X	0.1051045 (1)
bacteriocin	12	No live *A. salina* nauplii were found.				
bacterium	06	337.2982	454.5214	250.3075	Y = -2.730795 + 3.05805 X	1.773749 (3)
bacterium	12	205.9645	295.8508	143.3878	Y = -1.064911 + 2.621199 X	0.6596413 (3)

DISCUSSIONS

Microbial antagonism is a biological phenomenon in which certain microorganisms of the normal microbiota suppress the growth of other microorganisms through competition for nutrients and the secretion of inhibitory substances. These substances may be toxic to other species. Induction of antibiotic production has also been shown for endosymbiotic bacteria and even for human pathogens such as *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Samac *et al*., 2003). Bacterial infection is one of the major disease problems in shell fish and fin fish aquaculture. Disease cause the largest economic losses in aquaculture and bacterial infections are second compared to fungal diseases in economic importance. Bacterial infections are generally restricted to chronic, steady losses (Intesar, 2003). Gram negative bacteria are cocci or coccibacilli whose associated disease usually involve the accumulation of copious amounts to pus frequently affect the respiratory tract. The bacteria causing the most serious diseases of the post larval and adult stages of *P. monodon* of the genus *Vibrio, Bacillus, Pseudomonas* and *Aeromonas* (Lightner and Redman, 1998). Usually in prawn culture water or even in entrophicated coastal water bacterial numbers are less than 10^6 cells /ml because protozoa feed on bacterial cells which results in maintaining a certain level of bacterial populations in water (Maeda and Nogami, 1989).

We have confirmed that the isolated *Pseudomonas aeruginosa* secrete toxic chemicals, which act on *Artemia salina* as a lethal substance. Narasimhan *et al.* (2013) also reported that *Pseudomonas aeruginosa* is a pathogenic bacteria for prawn. So far, the isolated bacterial concentration in pond water or in aquatic organism can severely affect *Penaeus*

monodon at mentioned LC_{50} range. In case of bacteriocin the LC_{50} was 65.65591 µl and the regression equation was Y = 2.166407 + 1.559255 X, while the 95% confidence limits are 25.40323 to 69.691 µl for 6h exposure only. The cytotoxic effect obtained from the probit mortality analysis emphasis about the isolated bacterial toxicity on *Penaeus monodon* and other aquatic organisms.

ACKNOWLEDGEMENTS

Ministry of science and technology, govt. of the people's republic of Bangladesh is gratefully acknowledged for funding this research.

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